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(57) Abstract

Fusion polypeptide comprising an antibody binding domain and an HIV-derived epitope are disclosed. Also disclosed are compositions containing the fusion polypeptide and methods of use.

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HIV FUSION POLYPEPTIDE

BACKGROUND

1. Field of the Invention

This invention relates generally to the use of recombinant DNA technology in the development of antigen specific polypeptides, and specifically to a fusion HIV/anti-MHC or HIV/anti-IgD polypeptide, which binds to a receptor on antigen presenting cells and can be used to induce an immune response to HIV.

2. Description of the Prior Art

Due both to the major histocompatibility complex (MHC)-restricted nature of the T-cell response to antigen and to the relative paucity of T-cell epitopes in protein antigens, the effective use of synthetic peptide vaccines has been difficult in outbred populations such as humans. Therefore, any strategy that could overcome MHC-determined unresponsiveness or potentiate the immune response to subdominant epitopes would greatly improve the practical value of synthetic peptides as vaccines.

Synthetic peptides having pathogen-derived T and B cell epitopes can function as complete immunogens that elicit neutralizing antibodies and T cell memory. Thus, in theory at least, such peptides would be ideal as vaccines. The use of such peptides is limited, however, because of the MHC-restricted nature of the T cell response and their inherently weak immunogenicity.

Over the years, various attempts have been made to chemically couple proteins or polypeptides to antibody molecules or fragments in order to achieve site specific delivery to the antignic target bound by the antibody. The

selection of optimal conjugation conditions for preparing antibody-polypeptide conjugates varies for different antibodies, especially when monoclonal antibodies are used.

A major problem with prior techniques which utilize chemical coupling in attaching a polypeptide to an antibody is the difficulty associated with achieving consistent and reproducible results. Variables which impact on these difficulties include: (a) the site(s) of antibody attachment to the polypeptide, (b) the number of polypeptides attached to each antibody molecule, and (c) the possible loss of antibody binding activity or (d) a change in immunogenicity of the polypeptide following coupling. As a consequence of the difficulties associated in controlling these variables, classical techniques for coupling polypeptide to antibody require that each batch of polypeptide-coupled antibody be stringently monitored to account for these factors.

One possible approach to overcoming the problems related to classical chemical coupling of polypeptide to antibody would be to produce the antibody and polypeptide as a recombinant fusion polypeptide wherein the immunogenic polypeptide is operatively linked to the antibody. Such an approach would provide a population of fusion polypeptides which are homogenous with respect to the site of attachment of the immunogenic polypeptide to the antibody molecule, the number of polypeptides attached to the antibody and the activity of the antibody. The present invention provides such fusion polypeptides.

Optimally, a vaccine contains immunodominant antigens which elicit an effective immune response. At present, researchers have not been able to produce such a vaccine for AIDS, synthetic or otherwise. Acquired immune deficiency syndrome (AIDS) is a form of immunodeficiency that results from infection with a lymphocytotropic virus called human immunodeficiency virus (HIV). HIV

infection can cause profound lymphopenia, primarily of the CD4 subset of T lymphocytes. Affected individuals have decreased or absent delayed-type hypersensitivity, extreme susceptibility to opportunistic infections and may acquire certain unusual malignancies such as Kaposi's sarcoma or Burkitt's lymphoma. HIV also causes polyclonal expansion of B lymphocytes, leading to hypergammaglobulinemia. Despite the marked increase in amounts of immunoglobulins in serum, affected individuals are incapable of mounting a primary immune response to newly encountered antigens. The syndrome has been recognized primarily in "at risk" groups, including homosexually active males, intravenous drug users, recipients of blood or blood products, and certain populations from Central Africa and the Caribbean. The syndrome has also been recognized in heterosexual partners of individuals in all "at risk" groups and in infants of affected mothers. AIDS is almost invariably fatal.

The development of a vaccine against HIV is a critical step in preventing further spread of AIDS. For safety reasons, a whole virus vaccine may not be practical in the case of HIV. A vaccine should contain immunodominant helper T-cell sites, the distinct sites within a protein molecule that are recognized by the T-cells, that could elicit helper T-cell immunity in response to exposure to the native antigen. Hence, in order to elicit an effective immunogenic response, the antigen should include the immunodominant regions of the molecule.

SUMMARY OF THE INVENTION

This invention provides a fusion polypeptide molecule which comprises an antibody binding domain and an HIV immunogenic peptide. Preferably the antibody binding domain is anti-MHC or anti-IgD monoclonal antibody (mAb) Fab and the immunogenic peptide is an immunodominant HIV-1 epitope such as the V3 loop region. The Fab/HIV polypeptide is a hybrid fusion protein capable of presenting the HIV epitope to antigen presenting cells (APCs). The polypeptide consists of antibody Fab fragments expressing at their C-terminus immunogenic HIV-1 peptides. This invention provides the advantage of increasing immunogenicity and inherently providing adjuvant activity by focusing HIV antigenic epitopes at high density on the surface of APCs. The invention also provides polynucleotide sequences encoding the fusion polypeptide monoclonal antibody. These recombinant antibody genes provide a renewable, reproducible source of antibodies which can be further engineered to alter affinity constants and effector functions, thereby producing a highly effective composition capable of inducing an immune response to HIV.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a schematic illustration of the recombinant pComb3 vector system used for the expression of the hybrid fusion Fab fragments in *E. coli* XL-1 Blue.

FIGURE 2 shows Western blot analysis of fusion polypeptide 25-9-17SII Fab.

FIGURE 3 shows immunofluorescence and FACS analysis of binding of hybrid fusion Fab-fragments to their antigen displayed on the cell surface.

FIGURE 4 shows immunogenic targeting of HIV-1 hybrid fusion peptide vaccines.

DETAILED DESCRIPTION

The present invention provides a fusion polypeptide that comprises a first polypeptide which binds to a surface determinant of an antigen presenting cell (APC) and a second peptide which is an HIV-derived epitope. The fusion polypeptide stimulates an immune response (e.g., neutralizing antibodies, cell-mediated immunity, and immunological memory) to HIV-1 and provides inherent adjuvant activity. The invention also provides polynucleotide sequences which encode the fusion polypeptide. The pharmaceutical compositions of the invention contain the fusion polypeptide, and can be used to induce an immune response to HIV.

As used herein, "polynucleotide" refers to a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger construct. DNA encoding the hybrid polypeptide of the invention can be assembled from cDNA fragments or from oligonucleotides which provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit. Polynucleotide sequences of the invention include DNA, RNA and cDNA sequences.

As used herein "fusion polypeptide" refers to an antibody binding domain, such as a monoclonal antibody, which is antigen specific and is encoded by a polynucleotide sequence which contain sequences that do not occur contiguously in the natural environment. The Fab portion of the monoclonal antibody of the invention may be, for example, a monoclonal antibody for MHC determinants or other surface antigen determinants of APCs. The Fab fragment is fused to an HIV-derived epitope, for example, from the V3 loop region of gp120. The polynucleotide sequence for the Fab portion of the monoclonal antibody of interest can be the isolated from the hybridoma

producing that monoclonal antibody by methods well known in the art (Kohler and Milstein, *Nature*, 256:495, 1975).

The term "antibody binding domain" as used in this invention means heterodimeric molecules which are capable of binding to an epitope present on the APC. Such binding domains include intact antibody molecules, as well as fragments thereof, such as Fab and $F(ab')_2$, which retain the ability to bind an epitopic determinant and are capable of focusing antigenic epitopes at high density on the surface of antigen presenting cells.

As used herein, "antigen-presenting cell" refers to cells, such as dendritic cells, Langerhans cells, and mononuclear phagocytes that help in the induction of an immune response by presenting antigen to helper T lymphocytes. B lymphocytes can assume the function of accessory cells in antigen presentation.

"Major histocompatibility complex" (MHC) refers to the chromosomal region containing genes that encode cell surface glycoproteins that regulate interactions among cells of the immune system. The MHC typically contains genes encoding class I histocompatibility molecules and class II histocompatibility molecules, which are important for the growth and differentiation of B and T lymphocytes, antigen presentation, cytotoxicity, graft rejection and mixed lymphocyte reactions. Monoclonal antibodies against surface determinants of the invention preferably bind to MHC class I and/or MHC class II. Alternatively, such surface determinants as immunoglobulins can be utilized as binding targets of the antibody binding domain portion of the fusion polypeptide. Most preferably, IgD immunoglobulin, a predominant surface component of B lymphocytes, is used. Other APC surface determinants known in the art can also serve as binding targets.

"HIV-derived epitope" refers to an antigenic determinant that has been derived from the human immunodeficiency virus (HIV). The preferred epitope of the invention is derived from the envelope glycoprotein, gp120, of the HIV-1 virus. An epitope usually consists of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Antigenic variability is a main obstacle to HIV vaccine development. The main HIV-1 neutralization epitope is located in the hypervariable region of the envelope (*env*) gene, referred to as V3. This epitope occurs as a disulfide-bridged loop containing 35 amino acids with a beta-turn sequence, Gly-Pro-Gly in its center. Preferably, the epitope of the invention is a 15-amino acid peptide (residues 315-329) contained within the immunodominant V3 loop region of gp120.

The HIV-derived epitope of the invention is preferably from the isolate HIV type 1 (HIV-1). At present there are two known HIV isolates, HIV-1 and HIV-2. While both are related, the virus primarily responsible for the AIDS epidemic is HIV-1. The two viruses are similar in their overall structure and both can cause AIDS, although the pathogenic potential of HIV-2 is not as well established. In addition, HIV-2 is primarily found in West Africa, whereas HIV-1 is concentrated in central Africa and other regions of the world.

Preferably, the antibody binding domain produced according to the invention is heterodimeric and is, therefore, normally comprised of two different polypeptide chains, which together assume a conformation having a binding affinity, or association constant for the APC surface determinant. The binding affinity of the cloned antibody binding domain may be different, preferably higher, than the affinity or association constant of either of the polypeptides alone (i.e., as monomers). For example, where one or both of the different polypeptide chains is derived from the variable region of the light and heavy

chains of an immunoglobulin, polypeptides comprising the light (V_L) and heavy (V_H) variable regions are employed together for binding the APC surface determinant.

An antibody binding domain produced according to the invention can be active in monomeric as well as multimeric forms, either homomeric or heteromeric, preferably heterodimeric. For example, V_H and V_L ligand binding polypeptide produced by the present invention can be combined in the heterodimer to modulate the activity of either, or to produce an activity unique to the heterodimer. The individual antibody binding domain polypeptides are referred to as V_H and V_L and the heterodimer is referred to as a Fv.

However, it should be understood that a V_H binding polypeptide may contain, in addition to the V_H, substantially all or a portion of the heavy chain constant regions. A V_L binding polypeptide may contain, in addition to the V_L, substantially all or a portion of the light chain constant region. A heterodimer comprising a V_H binding polypeptide containing all or a portion of the first heavy chain constant region and a V_L binding polypeptide containing substantially all of the light chain constant region is termed an Fab fragment. The production of Fab can be advantageous in some situations because the additional constant region sequences contained in an Fab, as compared to a F_V, may stabilize the V_H and V_L interaction. Such stabilization could cause the Fab to have higher affinity for antigen.

The individual V_H and V_L polypeptides will generally have about 125 or fewer amino acid residues, more typically fewer than about 120 amino acid residues. Normally, the V_H and V_L polypeptides will have greater than about 60 amino acid residues, usually greater than about 95 amino acid residues, more usually greater than about 100 amino acid residues. Preferably, the V_H will be from

about 110 to about 125 amino acid residues in length while V_L will be from about 95 to about 115 amino acid residues in length.

The amino acid residue sequences of the V_H and V_L polypeptides will vary widely, depending upon the particular immunoglobulin idiotype involved. Usually, there will be at least two cysteines separated by from about 60 to 75 amino acid residues and joined by a disulfide bond. The antibody binding domain polypeptides produced according to the present invention will normally be substantial copies of idiotypes of the variable regions of the heavy and/or light chains of immunoglobulins, but in some situations a polypeptide may contain random mutations in amino acid residue sequences in order to improve the desired activity.

In some situations, it may be desirable to provide for covalent cross linking of the V_H and V_L polypeptides, which can be accomplished by providing cysteine residues at the carboxyl termini or by joining the V_H and V_L polypeptides by a synthetic linker. The polypeptide will normally be prepared free of the immunoglobulin constant regions, however a small portion of the J region may be included by selecting the appropriate DNA synthesis primers. The D region will normally be included in the transcript of the V_H.

Typically, the C terminus region of the V_H and V_L polypeptides will have a greater variety of sequences than the N terminus and can be further modified to permit modification of the normally occurring V_H and V_L chains. A synthetic polynucleotide can be employed to vary one or more amino acid residues in a hypervariable region.

The vector of the invention can be used to produce fusion polypeptides which are composed of more than one polypeptide chain. In such circumstances, the present invention contemplates embodiments in which one or more of the

antibody binding domain polypeptide chains is operably linked to a peptide or polypeptide derived from HIV. In a preferred embodiment, in which the antibody binding domain is specific for MHC or IgD, the antibody binding polypeptide of heavy chain, the antibody binding polypeptide of light chain, or both heavy and the light chains can be operably linked to at least one peptide of HIV-1.

The antibody binding domain of the invention can be derived from mammalian DNA or RNA isolated from lymphocytes, DNA or RNA isolated from hybridoma cell lines, or any other lymphocytes cultured *in vitro*, as well as previously cloned antibody fragments. Those of skill in the art will appreciate that the DNA sequences encoding the antibody binding domain can be altered by *in vitro* methods, including site-directed and random mutagenesis to alter the specificity and/or affinity of the encoded antibody binding domain for a an APC surface determinant.

A preferred method for the identification and isolation of antibody binding domain which exhibit binding with the APC surface determinant is the bacteriophage λ vector system. This vector system has been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in *Escherichia coli* (Huse, *et al., Science*, 246:1275-1281, 1989) and from the human antibody repertoire (Mullinax, *et al., Proc. Natl. Acad. Sci.*, 87:8095-8099, 1990). As described therein, receptors (Fab molecules) exhibiting binding for a preselected ligand were identified and isolated from these antibody expression libraries. This methodology can also be applied to hybridoma cell lines expressing monoclonal antibodies with binding for a preselected ligand. Hybridomas which secrete a desired monoclonal antibody can be produced in various ways using techniques well understood by those having ordinary skill in the art and will not be repeated here. Details of these

techniques are described in such references as *Monoclonal Antibodies-Hybridomas: A New Dimension in Biological Analysis*, Edited by Roger H. Kennett, *et al.*, Plenum Press, 1980; and U.S. 4,172,124.

Many hybridomas and their corresponding monoclonal antibodies are available for the preparation of DNA sequences encoding the antibody binding domain. DNA or RNA encoding the antibody binding domain can be obtained from a wide range of hybridomas (*ATCC Cell Line and Hybridomas*, American Type Culture Collection, Rockville, MD, 1988). Hybridomas secreting monoclonal antibodies which react with various APC surface determinants are listed in this reference and are readily available from the collection and usable in the invention.

DNA sequences encoding antibodies which bind surface determinants of the invention can be obtained by several methods. For example, the DNA can be isolated directly from hybridoma cell lines. Alternatively, hybridization procedures which are well known in the art can be utilized. These include, but are not limited to: 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences and 2) antibody screening of expression libraries to detect shared structural features.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. For example, oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide, stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. Hybridization procedures are useful for the screening of recombinant clones by using mixed synthetic oligonucleotide probes where

each probe is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucleic Acid Research*, 9:879, 1981).

The development of specific DNA sequences encoding antibody binding domain to surface antigens of APCs and the HIV epitopes can also be obtained by : 1) isolation of double-stranded DNA sequence from a genomic library; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

Of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures, the use of genomic DNA isolates (1), is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides because of the presence of introns.

The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is

known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the formation of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of cDNA libraries (plasmid or phage), which are derived form reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al., Nucleic Acid Research*, 11:2325, 1983).

Polynucleotide sequences encoding the hybrid antibody can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the hybrid antibody of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

Minor modifications of the recombinant hybrid antibody primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the antibodies described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All antibodies produced by these modifications are included herein as long as the immunogenic activation with respect to HIV and surface antigen recognition by APCs exists.

In the present invention, polynucleotide sequences encoding the fusion polypeptide may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the chimeric antibody genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted

genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells.

Methods for the random combination of the heavy and light chain antibody fragments include a combinatorial method in which the heavy and light chain antibody fragments are ligated into separate expression vectors and the vectors then joined at a common combining site to generate a single vector capable of expressing both heavy and light chain antibody fragments (Huse, *et al., Science*, 246:1275-1281, 1989; Mullinax, *et al., Proc. Natl. Acad. Sci.*, 87:8095-8099, 1990; Huse, WO 90/14443). Alternatively, the heavy and light chain antibody fragments can be joined at a common combining site, such as a common restriction site, before insertion into one expression vector. The common combining site can be incorporated into the heavy and light chain antibody fragments through a polymerase chain reaction (PCR) in which the primers contain the common restriction site and thereby incorporate the site into the heavy and light chain antibody fragments. In addition, the heavy and light chain antibody fragments can be joined by using fusion-PCR in which the heavy and light chain antibody fragments are amplified in a PCR reaction using primers which contain common complementary sequences before insertion into a single expression vector. Those of skill in the art will know of other methods for combining antibody fragments, or can readily ascertain such methods without undue experimentation.

Other techniques for amplifying nucleic acid can also be used to clone the hybrid monoclonal antibody polynucleotide of the invention in a single step. An example of another technique is self-sustained sequence replication, 3SR, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA.

Another method nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin with either DNA or RNA and finish with either, and amplifies to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single- stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to the promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9-fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest. Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligo probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for *HincII* with a short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. *HincII* is added but only cut the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the site of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7-fold amplification in 2 hours at 37°C. Unlike PCR

and LCR, SDA does not require instrumented temperature cycling. Although PCR is the preferred method of amplification of the invention, these other methods can also be used to clone the fusion polynucleotide of the invention.

The efficiency of utilizing antibody binding domain DNA for inclusion in the vectors of the invention can be enhanced by producing multiple copies of the DNA. This can be achieved by amplification of the antibody binding domain DNA sequence through the use of technology such as PCR. PCR technology can be used for any antibody binding domain where the appropriate oligonucleotide primers have been identified. (Lerner and Sorge, WO 90/14430; Sastry, *et al.*, *Proc. Nat. Acad. Sci. U.S.A.*, 86:5728, 1989; Orlandi, *et al., Proc. Nat. Acad. Sci. U.S.A.*, 86:3833, 1989).

In general, expression vectors for the production of fusion polypeptides of the invention contain replicon and control sequences which are derived from species compatible with a host cell for use in connection with the host. The vector typically carries a replicon site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. The vector must also contain, or be modified to contain, promoters which can be used by the host for expression. Promoters which are commonly used in recombinant DNA construction include the β-lactamase lactose promoter systems, λ phage promoters, and the tryptophan promoter systems. Other promoters have been discovered and can also be utilized.

The expression vector of the fusion polypeptide can also be placed under control of other regulatory sequences which may be homologous to the host in its untransformed state. For example, lactose dependent *E. coli* chromosomal DNA comprises a lactose operon (*lac*) which mediates lactose utilization by elaborating the enzyme β-galactosidase. The *lac* control elements may be obtained from bacterial phage λ plac 5 which is infective for *E. coli*.

The *lac* promoter-operator system can be induced by isopropy-β-thio-galactopyranoside (IPTG).

Other preferred hosts for expression of the vectors of the invention are mammalian cells, grown *in vitro* in tissue culture or *in vivo* in animals. The mammalian cells provide post translational modifications to eukaryotic protein molecules which includes correct folding or glycosylation at specific sites. Mammalian cells which may be useful as hosts include cells of fibroblast origin such as VERO or CHO cells, or cells of lymphoid origin, such as the hybridoma SP2/0-AG14 or the myeloma P3x63Ag8, and their derivatives.

Several vector systems are readily adaptable for use in expressing the cloned fusion polypeptide in mammalian cells. One class of vectors utilizes DNA elements which provide autonomously replicating extra chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, or SV40 virus. A second class of vectors utilizes integration of the desired gene sequences into the host chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing drug resistance genes, such as *E. coli* GPT or Tn5neo. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of fusion receptor protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements are available to those of skill in the art (Okayama, *Molec. Cell. Biol.*, 3:280, 1983).

Another host which can be suitably utilized for expression of the DNA sequences of the invention is yeast. Yeast provide substantial advantages in that host translational polypeptide modification can also be carried out in these

organisms. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number vectors which can be utilized for producing the fusion receptor protein in yeast. Since yeast recognize leader sequences on cloned mammalian gene products, peptides bearing leader sequences will be secreted in these systems.

The fusion polypeptide of this invention can be used as reagent for prevention or immunotherapy of HIV infections. The clinical utility of the invention is supported by the specificity of the fusion polypeptide for HIV, especially gp120, V3 loop, a virtually limitless supply of fusion polypeptides, and its ability to evoke a specific immune response to HIV, thus overcoming major drawbacks of polyclonal antibodies.

A pharmaceutical composition in accordance with the present invention contains at least one dose of fusion polypeptide effective in inducing an immune response to HIV. The fusion polypeptide can be packaged and sold in freeze-dried or other acceptable form, and/or mixed with a therapeutically acceptable carrier, such as a balanced aqueous salt solution.

An immunotherapeutic method in accordance with this invention entails the administration of the fusion polypeptide of the invention by injection or infusion prior to (prophylaxis) or following (therapy) the onset of infection with HIV. The amount of fusion polypeptide required to induce an immune response to HIV depends on such factors as the type and severity of the infection, the size and weight of the infected subject, and the effectiveness of other concomitantly employed modes of prophylaxis or therapy.

The immunotherapeutic method of the invention includes a prophylactic method directed to those humans at risk for HIV infection. A "prophylactically effective" amount of the pharmaceutical composition containing the fusion polypeptide

refers to that amount which is capable of inducing an immune response to HIV. Transmission of HIV occurs by at least three known routes: sexual contact, blood (or blood product) transfusion and via the placenta. Infection via blood includes transmission among intravenous drug users. Since contact with HIV does not necessarily result in symptomatic infection, as determined by seroconversion, all humans may be potentially at risk and therefore should be considered for prophylactic treatment with the fusion polypeptide of the invention.

The fusion polypeptide of the invention can be administered parenterally by injection or by gradual infusion over time. For example, the fusion polypeptide can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally, alone or in combination with effector cells.

Preparations for parenteral administration are contained in a "pharmaceutically acceptable carrier". Such carriers include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents include propylene glycol, polyethylene glycol, metabolizable oils such as, olive oil, squalene or squalane, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The term "therapeutically effective" means that the amount of hybrid polypeptide used is of sufficient quantity to increase the subject's immune response to HIV. The dosage ranges for the administration of the monoclonal antibody of the invention are those large enough to produce the desired effect in which the HIV epitopes are focused on the surface of the APCs, thereby allowing a more efficient antigen presentation and therefore a more effective vaccination. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one skilled in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. In any event, the effectiveness of treatment can be determined by monitoring the level of CD4+ T-cells in a patient. An increase in CD4+ cells should correlate with recovery of the patient's immune system.

The pharmaceutical composition of the invention can be administered to a patient prior to infection with HIV (i.e., prophylactically) or at any of the stages described below, after initial infection. The HIV infection may run any of the following courses: 1) approximately 15% of infected individuals have an acute illness, characterized by fever, rash, and enlarged lymph nodes and meningitis within six weeks of contact with HIV. Following this acute infection, these individuals become asymptomatic. 2) The remaining individuals with HIV infection are not symptomatic for years. 3) Some individuals develop persistent generalized lymphadenopathy (PGL), characterized by swollen lymph nodes in the neck, groin and axilla. Five to ten percent of individuals with PGL revert to an asymptomatic state. 4) Any of these individuals may develop AIDS-related complex (ARC); patients with ARC do not revert to an asymptomatic state. 5) Individuals with ARC and PGL, as well as asymptomatic individuals, eventually (months to years later) develop AIDS which inexorably leads to death.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

The system for generating combinatorial Fab cDNA libraries in modified lambda ZAPphage vectors which express their products in *E. coli* (Huse, *et al.*, *Science*, 246:1275-1281, 1989) was used to clone antigen-binding Fab fragments from anti-MHC antibody-secreting hybridomas. Hybrid primers that contained, in addition to immunoglobulin-specific sequences, nucleotide sequences encoding the HIV-1 loop peptide, as well as restriction sites for directional cloning were designed in order to amplify and clone in a single step chimeric Fab fragments expressing the V3 loop peptide. These primers, together with cDNA templates reverse-transcribed from hybridoma-derived mRNA, were used in polymerase chain reactions. The reactions yielded cDNA fragments of expected size from all hybridomas tested. The identity of the amplified PCR products was confirmed by Southern blotting using internal oligonucleotide probes specific for light chain/kappa or heavy chain Fd fragments. The Fd refers to the V^H plus CH^1 regions of the heavy chain. These PCR-amplified chimeric fragments were directionally cloned into the modified lambda ZAP vectors, lambda Lc1 and lambda Hc2 respectively. Lκ and Fd fragment-encoding phage DNAs were combined to create a bicistronic construct, able to express monovalent Fab fragments in *E. coli*. The desired clones were then screened and selected for the presence of both Lκ and Fd-encoding cDNA fragments using appropriate oligonucleotide probes and replica hybridization techniques. Positive lambda phage were plaque-purified, and phagemids were excised in *E. coli* using the M13 helper phage.

Sequence analysis of plasmid DNA indicated that hybrid hybridoma-derived Fab fragments were correctly cloned.

EXAMPLE 1

The HIV epitope chosen initially for expression in hybrid Fab constructs is a 15-amino acid peptide, NH_2-RIQRGPGRAFVTIGK-COOH, representing amino acids 315-329 in the V3 loop region of the envelope glycoprotein, gp120, derived from the IIIB isolate of HIV-1. This well characterized region represented a good model antigen because it was reported to contain a neutralizing antibody epitope, as well as epitopes recognized by MHC-restricted T helper cells and cytotoxic T cell. As targeting antibodies, several mAb-producing hybridomas, obtained from ATCC or the originating laboratories, specific for murine or human MHC class II or sIgD molecules were chosen, and are listed below:

	ATCC#
L203 (Anti-Human HLA-Dr, monomorphic determinant), mouse IgG, γ1,κ	HB 171
IA6-2 (Anti-Human δ-chain), mouse IgG, γ2a, κ	--
11-26 (Anti-Mouse IgD), rat IgG, γ2a, κ	--
$H\delta_a/1$ (Anti-Mouse IgD^a), mouse IgG, γ2b, κ	--
AF3.33 (Anti-Mouse IgD^b), mouse IgG, γ2a, κ	--
B21-2 (Anti-Mouse I-$A^{b,d}$), rat IgG, γ2b, κ	TIB 229
BP107.2.2 (Anti-Mouse I-$A^{b,d,j,p,q,u}$) mouse IgG, γ3, κ	TIB 154
MK-D6 (Anti-Mouse I-A^d) mouse IgG, γ2a, κ	HB3

EXAMPLE 2

Cloning and Expression of Chimeric Fab on the Phage Surface

To clone antigen-binding Fab fragments, a recently described procedure for assembling combinatorial, antigen-binding Fab libraries on phage surfaces, using the modified phagemid vector, pComb3 (Barbas III, *et al., Proc. Natl. Acad. Sci., USA*, 88:7978, 1991) was used. The power of this cloning method derives from the ability to readily screen very large libraries ($\geq 10^{12}$ clones) and to select and enrich phage particles expressing the desired Fab by affinity purification on antigen-coated plates.

A. RNA Isolation and cDNA Synthesis

Total RNA from hybridoma cell lines was isolated by the RNAzol B-method (Cinna/Biotecx, TX). In general, 10^6 cells were lysed in 100 μl of RNazol B solution, followed by phenol/chloroform extraction and isopropanol precipitation according to the manufacturer's protocol. Two μg total RNA was used as template for oligo(dT) (Lκ cloning)- or C_{H2} (Fd cloning)-primed first strand cDNA synthesis using Superscript RNase H reverse transcriptase (Gibco-BRL, MD) by the standard method.

B. DNA-Amplification Using Ig-consensus PCR-primers

Chimeric Fab-encoding cDNAs were amplified in standard polymerase chain reactions. The various PCR primers and oligonucleotide probes are listed below:

Oligonucleotide Primers and Probes:

cDNA-consensus primer

1. CRP_{H2}, γ1, γ2a, γ2b, γ3: 5'-ACCAC(C/A)ACACA(T/C)GTGA-3'

Heavy Chain PCR Primers

2. VRP_H 5'-AGGT(C/G)(C/A)A(G/A)CT(G/T)CTCGAGTC(T/A)GG-3'
3. CRP_{H1}, γ1 5'-TATGCAACTAGTAGATCTACAATCCCTGGGCACAAT-3'
4. CRP_{H1}, γ2a, γ2b, γ3 5'- ACAGGGACTAGTAGATCTGGGCACTCTGGG-CTCAATTTTCTT-3'

Light Chain PCR Primers

5. VRPκ 5'-CCAG(A/T)T(G/C)(T/C)GAGCTC(C/G)(A/T)(C/G)(C/A)T(C/G)A-C(C/A)CAG(A/T)CTCCA-3'
6. V3-CRPκ:5'-GCGCCGTCTAGAATTATTTTCCTAT-TGTAACAAATGCTCTCCCTGGTCCTCTCTGGATACGGGATCCACAC-TCATTCCTGTTGAAGCTCTTGAC-3'
7. ANP-Cκ:5'-GCGCCGTCTAGAATTATTTTCCTATTGTAACAAA-3'

Internal Oligonucleotide Hybridization Probes

8. Cκ-IP: 5'-ACTGCCATCAATCTTCCACTTGAC-3'
9. C_{H1}-IP: 5'-GAA(A/G)TA(G/A)CCCTTGACCAGGCATCC(C/T)AG-3'

V3-loop Tandem Encoding Oligonucleotides

10. V3-sense: 5'-GATCTGGTGGCGGTGGCTCTCTGCAGCGTATCCAGA-GAGGACCAGGGAGAGCATTTGTTACAATAGGAAAAGTCGAC-3'

11. V3-Antisense: 5'-CTAGCATTATTTTCCTATTGTAACAAATGCTCTCCC-TGGTCCTCTCTGGATACGGTCGACTTTTCCTATTGTAACAA-3'

-CRP, constant region primer; VRP, variable region primer; ANP, anchor primer. Underlined nucleotides represent restriction enzyme sites used for cloning.

The PCR mixture included ~0.5 μg cDNA template, 0.3 μg primers, all four dNTPs (200 μM each) in *Taq* polymerase buffer containing 1.5 mM $MgC1_2$ and 5 units of *Taq* polymerase (Promega) in a final volume of 100μl. PCRs were conducted on a thermal cycler (TwinBlock Thermal Cycler, Ericomp., CA). Primers for the amplification of mouse or rat heavy chain sequences (Fd) were VRP_H and the subclass-specific CRP_{H1}, and the reactions were performed for 30 cycles under the following conditions: 91°C for 1 min., 52°C for 2 min. and 72° for 1.5 min., followed by a final elongation of 15 min. at 72°C. In order to amplify and clone in a single step chimeric Lκ fragments encoding the HIV-1 V3 loop peptide, hybrid primers were designed containing Ig- plus V3 loop peptide-specific nucleotide sequences. They were VRPκ (5') and a mixture of 3' primers consisting of hybrid primer V3-CRPκ plus its corresponding ANP-Cκ primer at a molar ratio of 9:1. Temperature cycles were as follows: 5 cycles of 91°C for 1 min., 45°C for 2 min. and 72°C for 1.5 min., followed by 25 cycles of 91°C for 1 min., 55°C for 2 min. and 72°C for 1.5 min., and a final elongation for 15 min. at 72°C. The identities of these PCR-amplified fragments were then confirmed by Southern hybridization techniques using internal oligonucleotide probes Cκ-IP and C_{H1}-IP.

C. Construction of Phage Display Libraries

Surface expression libraries were constructed in two steps. First, amplified Fd fragments were digested with an excess of restriction enzymes *XhoI/SpeI* at 37°C for 12 hours, and size-fractionated by agarose gel electrophoresis. DNA fragments were purified and directionally ligated in the *XhoI/SpeI*-linearized and phosphatase-treated pComb3 vector. Following transformation of *E. coli* XL1-Blue, 100 ml 2xTY medium containing ampicillin (100 μg/ml) was added and cultures were grown overnight at 37°C. Phagemid-DNA containing the Fd-

fragment library was isolated, linearized with *SacI/SpeI*, phosphatase-treated, and the recombinant 4.7 kb vector fragment was purified by agarose gel electrophoresis. Amplified Lκ fragments were treated similarly except that the restriction enzymes *SacI* and *XbaI* were used. The Lκ cDNA fragments were cloned in the Fd fragment-containing, *SacI/SpeI*-linearized pComb3-vector. Following transformation of *E. coli* (XL1-Blue), phage were generated as described by Barbas, *et al. (Proc. Natl. Acad. Sci., USA*, 88:7978, 1991).

D. Panning of the Combinatorial Library to Select Antigen Binders

Microtiter plates were coated with 1 μg purified antigens (MHC class II or IgD molecules) overnight at 4°C. The wells were blocked, incubated with the phage display libraries, washed and eluted with an 0.1 M glycine buffer, pH 2.5. The eluted phage were then used to infect *E. coli* XL1-Blue cultures. New phage stocks were prepared, and reincubated with antigen in microtiter wells as above. After three consecutive rounds of panning, bacterial colonies expressing chimeric Fabs of desired antigen specificity were detected in a filter assay in which bacterial colonies were grown for 16 hours at 37°C on Durapore master filters in close contact with a second membrane (Immobilon P) coated with anti-Ig antibodies. This was done on top of LB agar dishes containing 1 mM IPTG and 100 μg/ml ampicillin. Secreted bacteria-derived Fabs diffusing onto the second membrane were thereby immobilized by the capture antibodies and detected by specific binding to radiolabelled antigen (Dreher, *et al. J. Immunol. Meth.*, 139:197, 1991). Using this procedure, colonies expressing antibody fragments of the desired specificity could be easily identified and were picked for further analysis.

E. Preparation of soluble Fab fragments, containing three HIV-1 loop peptides

Phagemid DNA from positive clones was isolated and the *BglII-NheI* containing DNA-fragment encoding the phage coat protein, pIII, was replaced with an

artificial gene fragment encoding two tandem HIV-1 loop peptides plus convenient flanking restriction sites (Figure 1). The tandem loop-specific sequence was synthesized as two slightly overlapping single-stranded oligonucleotides (V3-sense and V3-antisense), kinased, annealed and ligated with *BglII-NheI*-linearized Fab phagemid DNA. Second strand was filled in with the Klenow fragment of DNA polymerase I before bacterial transformation, using standard procedures.

F. DNA Sequencing:

Plasmid DNAs from positive clones were sequenced by the dideoxy method using a Sequence 2.0 kit (USB Corp., OH), and analyzed by using MacVector sequence analysis software.

G. Western blot analysis

Mouse Fab fragments from IPTG-included bacterial cultures were separated in a nonreducing 12.5% SDS/polyacrylamide gel and immunostained with polyclonal anti-Ig antibodies, or with H902, an mAb specific for the HIV-1 loop epitope of the IIIB isolate, using standard procedures.

Western blot analysis was performed with 25-9-17SII (anti MHC class II I-A^d) Fab (Figure 2). An anti-gp120 mAb specific for the V3 loop peptide as well as polyclonal rabbit anti-mouse IgG antibodies reacted specifically with the expressed Fab fragments indicating that the corresponding HIV-1 epitope was also correctly expressed in the hybrid constructs.

H. Immunofluorescence and FACS analysis:

Binding of hybrid Fab to HLA-DR (MHC class II) molecules displayed on the surface of appropriate cell lines was evaluated by immunofluorescence and FACS-analysis. Bacterial clones were grown in 2xTY medium containing 100 μg/ml ampicillin and 20 mM $MgCl_2$ at 37°C until an OD_{600} of 0.8 was achieved.

1 mM IPTG was added and the culture was induced for 16 hours at 37°C. Cells were pelleted by centrifugation, resuspended in PBS and lysed by sonication on ice (3 min at 50% full power). Cellular debris were pelleted, and the cleared supernatant was directly used for immunofluorescence analysis. FITC-labeled anti-mouse or anti-rat $F(ab')_2$ antibodies were used as secondary reagents in standard staining procedures.

Recombinant hybrid Fab fragmnets retained binding to their antigen displayed on the cell surface with a similar intensity as native, hybridoma-derived intact antibody or Fab fragments (Figure 3). A human T cell leukemia line, HUT-78, displaying MHC class II molecules on its surface was stained with hybrid L203 Fab (a mouse anti-human MHC class II mAb) or its corresponding native antibody or Fab fragment (prepared by papain digestion), followed by a secondary FITC-anti-mouse IgG. The results showed that the hybrid antibodies bind to the cell surface antigen as well as intact, native antibody.

To show focusing of the HIV-1 derived loop epitope on the surface of human or primate lymphocytes by a hybrid anti-MHC class II Fab (L203), cells were stained with the L203 Fab, followed by biotin-labeled H902 (mAb specific for the HIV-1 loop epitope) and FITC-labeled streptavidin. Positive staining indicated the binding of the HIV-1 epitope to the cells (Figure 4). Anti-human MHC class II mAb L203) also showed binding to primate class II molecules. This showed the utility of these vaccines not only in mice (using anti-mouse MHC class II mAbs), but also in primate models of HIV-infection.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made without departing from the spirit or scope of the invention.

CLAIMS:

1. A fusion polypeptide comprising:
   a. a first polypeptide comprising an antibody binding domain which binds to a surface determinant on an antigen presenting cell (APC); and
   b. a second peptide which is an HIV-derived epitope.

2. The hybrid polypeptide of claim 1, wherein the surface determinant is selected from the group consisting of MHC class I, MHC class II, and immunoglobulin.

3. 3. The hybrid polypeptide of claim 2, wherein the immunoglobulin is IgD.

4. The hybrid polypeptide of claim 1, wherein the HIV-derived epitope is from HIV-1.

5. The hybrid polypeptide of claim 4, wherein the HIV-1 epitope is from gp120.

6. The hybrid polypeptide of claim 5, wherein the gp120 epitope is from the V3 loop region.

7. A polynucleotide sequence encoding the hybrid polypeptide of claim 1.

8. A recombinant expression vector containing the polynucleotide sequence of claim 7.

9. The vector of claim 8, wherein the vector is a virus.

10. The vector of claim 8, wherein the vector is a plasmid.

11. A host cell containing the vector of claim 8.

12. The host cell of claim 11, wherein the host cell is eukaryotic.

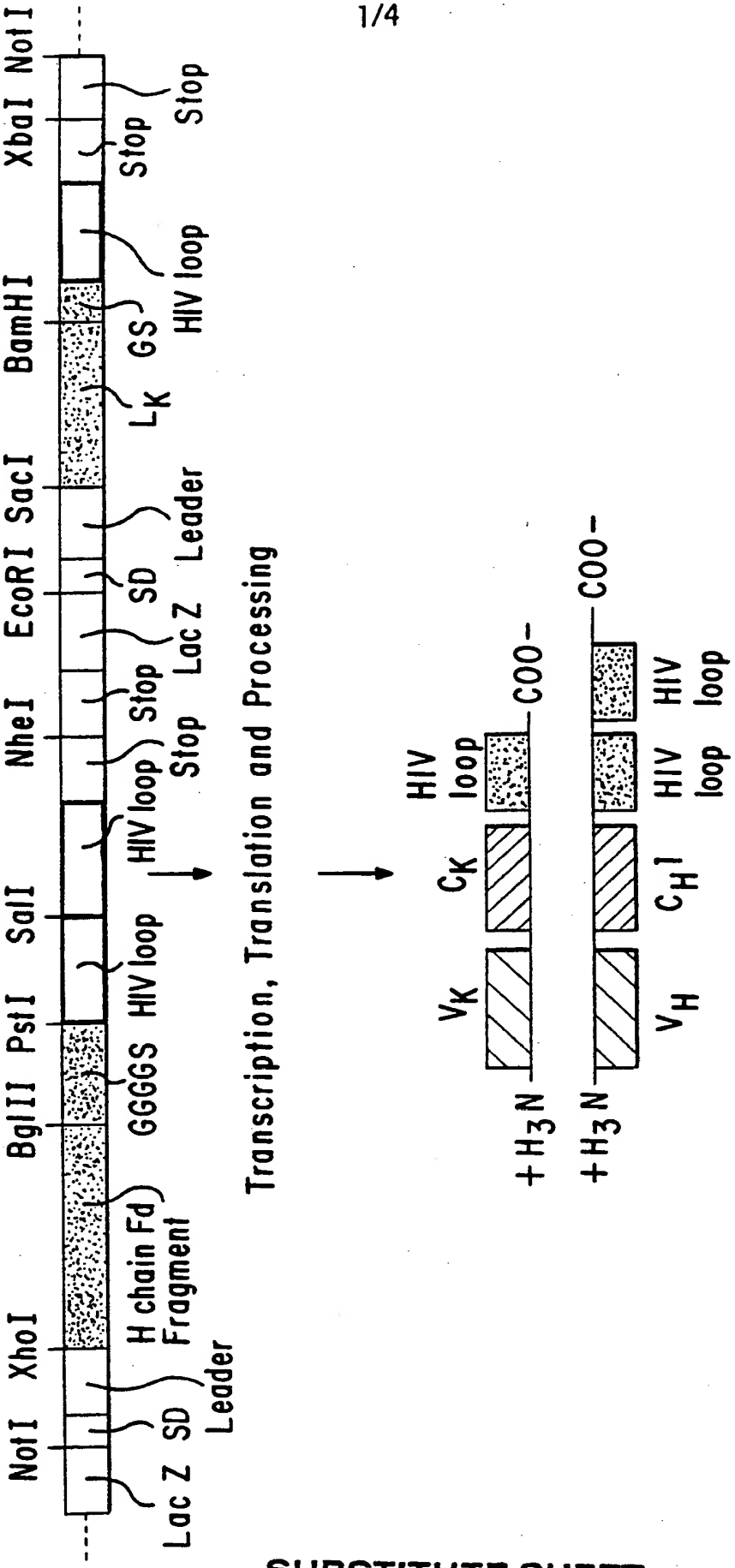
13. The host cell of claim 11, wherein the host cell is prokaryotic.

14. A pharmaceutical composition comprising at least one dose of the fusion polypeptide of claim 1, in a pharmaceutically acceptable carrier.

15. The pharmaceutical composition of claim 14, wherein the HIV is HIV-1.

16. A method of treating a patient having or at risk of having HIV infection comprising administering to the patient a therapeutically effective amount of the pharmaceutical composition of claim 14.

17. The method of claim 16, wherein the HIV is HIV-1.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H chain Fd Fragment
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L_K GS HIV loop
Stop Stop
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V_H C_H1 HIV loop HIV loop
$+H_3N$ COO-

FIG. 1

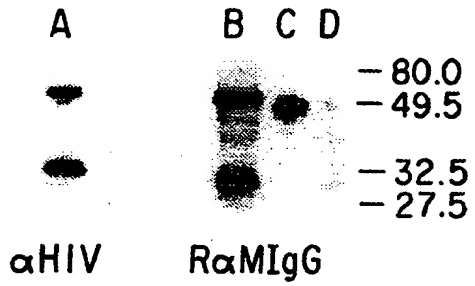
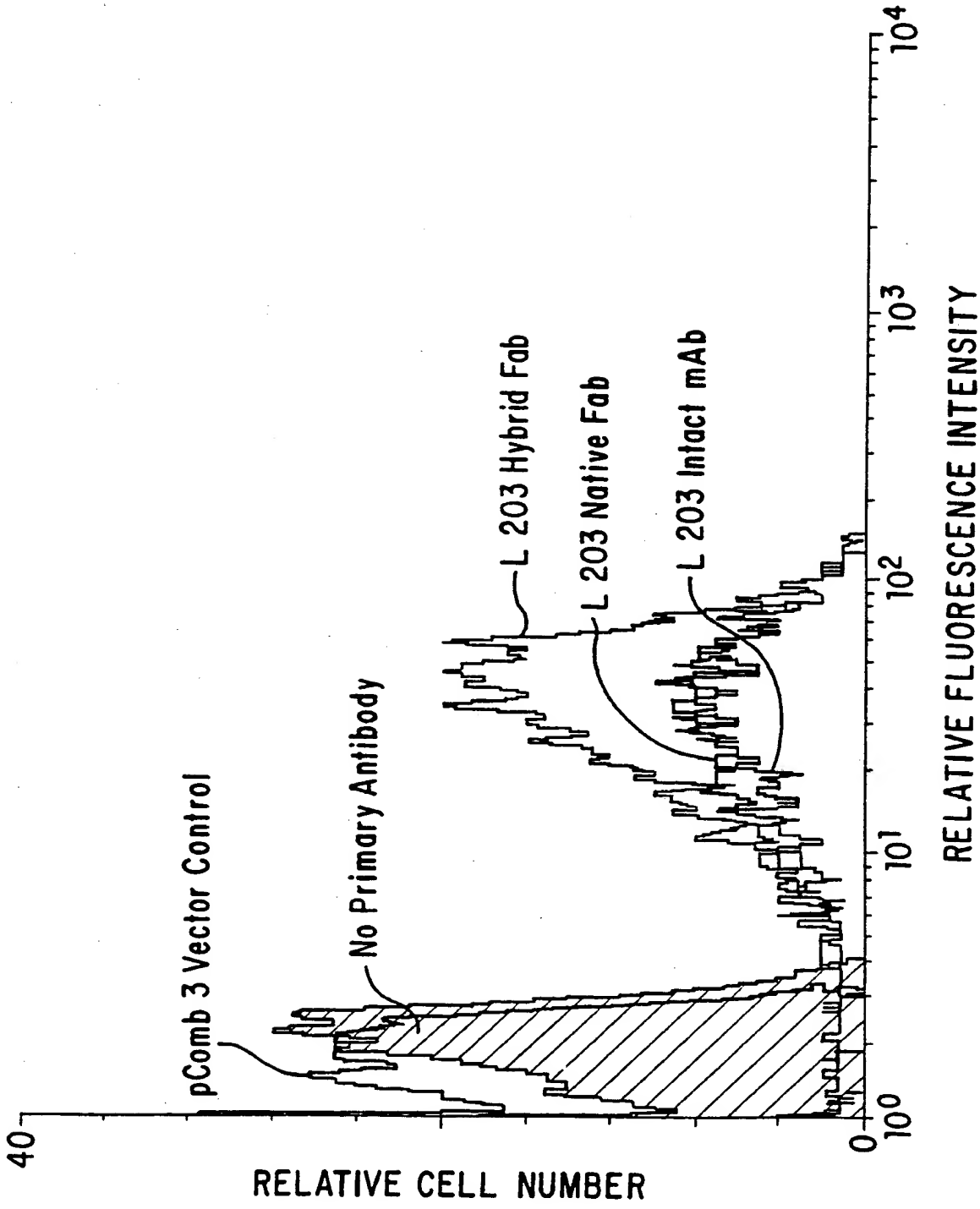


FIG. 2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pComb 3 Vector Control
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L 203 Native Fab
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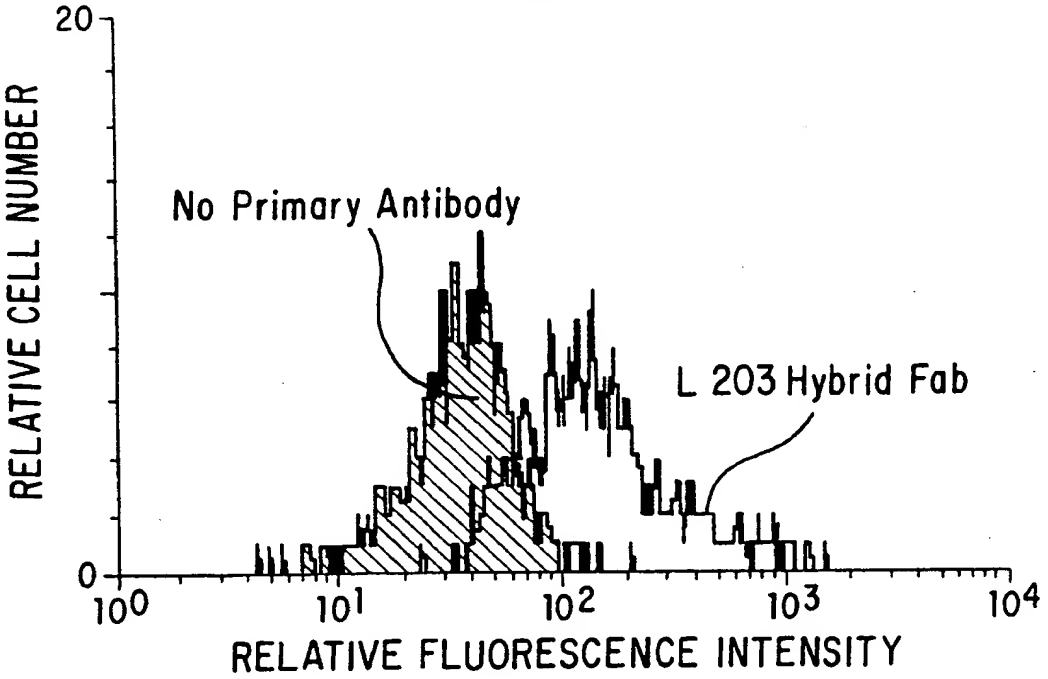
10^4
RELATIVE FLUORESCENCE INTENSITY

FIG. 3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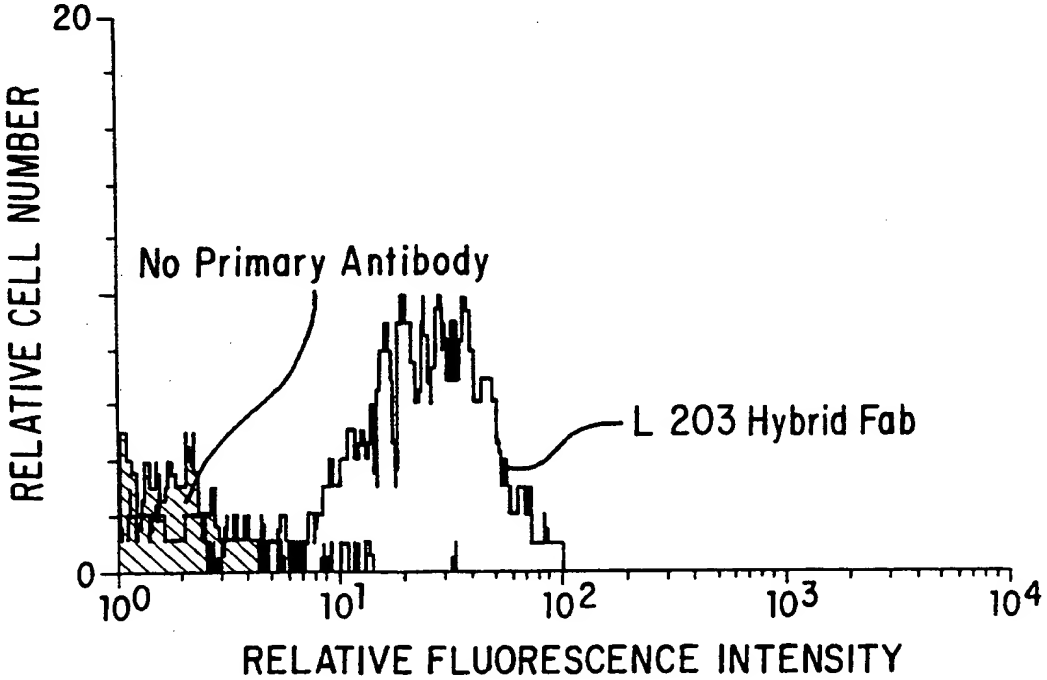
RELATIVE FLUORESCENCE INTENSITY

FIG. 4A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RELATIVE FLUORESCENCE INTENSITY

FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07966

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :U.S. CL: 530/395; 424/89; 536/27; 435/320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : U.S. CL: 530/395; 424/89; 536/27; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, APS, search terms: fusion polypeptide, HIV, variable loop, V3, immunoglobulin domain, class I, class II.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,116,964 (Capon et al) 26 May 1992, columns 2-8.	1-17
X	EP,A, 0,421,626 (Kniskern et al) 10 April 1991, pages 10-11.	1-17
X	EP,A, 0,314,317 (Capon et al) 03 May 1989, pages 2-5.	1-17
X	WO,A, 91/11454 (Sharma et al) 08 August 1991, pages 5-9.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 December 1992	Date of mailing of the international search report 21 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer LYNETTE F. SMITH Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07966

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K39/12; C12N 15/00; C07K 3/00, 17/00, 13/00, 15/00; C07H 15/12, 13/00